POTENTIAL APPLICATION OF PAPAIN ENZYME FROM PAPAYA LEAVES IN MAKING ANTIBACTERIA AND ANTIFUNGAL HANDWASH AND HAND SANITIZER

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ABSTRACT

This paper presents antibacterial and antifungal study of papain enzyme and its potential in making hand wash and hand sanitizer. Papain enzyme could be obtained from papaya latex, fruits and leaves. In this research, papaya leaves chose for the papain extraction because it is biomass and easily to get the source. The extraction method used in this research is hot water extraction follow by the Folin & Ciocalteu papain analysis method. Before making the antibacterial and antifungal handwash and hand sanitizer, antibacterial and antifungal test was done in order to proof the antibacterial and antifungal properties of papain enzyme. Antibacterial and antifungal test were done by incubating the pathogens on the nutrient agar in incubator for three days then insert papain enzyme onto it then incubate for another three days to determine the mycelial growth of pathogens. A volume of 187mL of papain solution was extracted by 21.55g and 200mL water. During the papain enzyme analysis, dark blue solution formed when Folin & Ciocalteu reagent was added into the solution sample. The blue colour indicate the present of tpapain enzyme. Enzyme concentration was measured in 0.415µm while the enzyme activity is 0.2283unit/mL. It means that 10mL of the papain solution extracted contains 2.283 unit of papain enzyme. The mycelial growth of the *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* bacteria and *Saccharomyces cerevisiae, Rhizopus spp. and Mucor spp*fungus were obviously decline 3 days after the papain solution added. Antibacterial and antifungal handwash and hand sanitizer were successfully produced at the end of this research.

ABSTRAK

Kajian ini membentangkan ciri-ciri anti-bakteria dan anti-kulat dalam enzim papain dengan potensi aplikasinya dalam pembuatan sanitizer tangan dan pencuci tangan. Enzim papain boleh didapati daripada 'latex' betik, buah dan daun betik. Dalam kajian ini, daun betik dipilih untuk pengekstrakan enzim papain kerana ia merupakan biojisim dan mudah didapati. Kaedah pengekstrakan yang diguna adalah 'Hot Water Extraction' diikuti dengan analisis papain 'Folin & Ciocalteu'. Sebelum membuat sanitizer tangan dan pencuci tangan, ujian anti-bakteria dan anti-kulat telah dijalankan untuk membuktikan ciri-ciri anti-bakteria dan anti-kulat yang terdapat dalam enzim papain. Ujian anti-bakteria and anti-kulat dijalankan dengan merangsangkan pathogen atas agar nutrien di dalam incubator selama tiga hari. Kemudian, enzim papain dimasukkan atas patogen dan dirangsangkan lagi selama tiga hari untuk menentukan pertumbuhan mycelial dalam patogen. Sebanyak 187mL larutan enzim papain telah diekstrak dari 200mL air. Semasa menjalankan analisis enzim papain, larutan biru tua menentukan larutan tersebut menpunyai komposisi enzim papain. Kepekatan enzim yang diukur melalui analisis enzim adalah 0.415µm dan aktiviti enzim pula ialah 0.2283unit/mL. Ini menunjukkan, dalam 10mL larutan enzim papain yang diekstrak, terdapat 2.283 unit enzim papain dalam larutan tersebut. Pertumbuhan mycelial *Staphylococcus aureus, Escherichia coli dan Bacillus subtilis bacteria dan Saccharomyces cerevisiae, Rhizopus spp. and Mucor sppfungus* telah menurun dengan jelas dari sudut pandangan penglihatan selepas tiga hari enzim papain ditambah. Anti-bakteria dan anti-kulat telah berjaya dibuat di akhir pengajian ini.

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LIST OF ABBREVIATIONS

AEBSF	4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride
ACN	Acetonitrile
BAEE	N-benzoyl-L-arginine ethyl ester
CATH	Class, architecture, topology, homologous superfamily
EDTA	Ethyl-diamine-tetra-acetic acid
E-64	Trans-Epoxysucciny-L-leucyl-amido(4-guanidino) butane; (L-3-trans-Carboxyoxiran-2-Carbonyl)-L-Leucyl-Admat
GMP	Good manufacturing practice
HCl	Hydrochloric acid
HWE	Hot water extraction
MAE	Microwave-assisted extraction
NaOH	Sodium hydroxide
PDE	Permitted daily exposure
PMSF	Phenylmethyl sulfonyl fluoride
PSE	Pressurized solvent extraction
SCFE	Supercritical fluid extraction
SCWE	Subcritical water extraction
SE	Soxhlet extraction
THF	Tetrahydrofuran
TLCK	Tosyllysine chloromethyl ketone hydrochlorid
TPCK	Technology pedagogical content knowledge
UV	Ultra violet

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Papaya belongs to *Carica*ceae family under kingdom Plantea. It originates from Mexico and South America. Papaya also called as pawpaw in Australia (Morton et al., 1987). The botanical name of papaya is *Carica* papaya. It is a small tropical tree with a straight stem marked by scars where leaves have fallen from it directly. There are many varieties of papaya, but the main varieties grown in the United State are Red Lady, Maradol, and various Solo types. According to Edison Frod (2011), to successfully grow a papaya tree, a frost free climate, lots of sunlight, lots of water and good soil needed. It is fast growing plant because it can grow up with fruits within 6 to 12 months.

Papaya fruits are melon-like, oval to nearly round, pyriform, or elongated club-shaped. They are 12-50cm long, and 10-20cm thick. The skin is waxy and thin but fairy tough. The fruit is varying size and weight, and can range from few 100g up to 10kg. (Augstburger et al., 2000). Christopher Columbus (2012) reputedly referred to the tropical fruit papaya as ‘fruit of the angels’. It is rich in vitamin A, E, K and B, fiber, calcium, magnesium, phosphorus and zinc, as well as the essential nutrients lycopene, folate, lutein and enzymes.

Carica papaya plants produce natural compounds (annonaceous acetogenins) in leaf bark and twing tissues. The leaves are large, usually are 50-70cm in diameter, deeply palmately lobed, with seven lobes. Papaya leaf is an herbaceous tree with a stem of spongy, soft wood that is hollow in the centre and bear melon-like fruit. Rajesh Sharma (2010) claims that papaya leaf is an excellent treatment for digestive disorders and extremely useful for any disturbance of the gastrointestinal tract. Efficacy papaya leaves has been widely used for traditional medicine in various countries particularly in a country where many overgrown papaya plants such as Indonesia, Vietnam and Australia. Although it is bitter, it is very good for the health of our bodies.

Several scientific investigations on the biological activities had been done through the leaves. Study shows the leaves are highly anti-humour and pesticidal properties. It was suggested that a potentially lucrative industry based simply on production of plant biomass could develop for production of anti-cancer drugs, pending Food and Drug Agency approval, and natural (botanical) pesticides (McLanghlin, 1992). The high level of natural self-defence compounds in the tree makes it high resistant to insect and disease infestation. (Peter, 1991). Atta, 1999 stated the fresh n green pawpaw leaf is an antiseptic, whilst the brown and dried pawpaw leaf is the best as a tonic and blood purifier. In addition, fresh and green papaya leaf has a therapeutic value due to its antiseptic quality. It cleans the intestines from bacteria. The tea made by pawpaw leaf promotes digestion and aids in treatment of ailments such as chronic indigestion, overweight and obesity, arteriosclerosis, high blood pressure and weakening of the heart.

Pawpaw leaf contains many active components that can increase the total antioxidant power in blood and reduce lipid peroxidation level, such as papain, chymopapain, crystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates. (Otsuki, N et al., 2010). In French Guiana, both leaf and root are prepared in combination with Quassia amara, Euterpe oleracea and Citrus species for the treatment of malarial fever. (Vigneron et al., 2005). According to the folk medicine, papaya latex can cure dyspepsia and also applicable for external burns and scalds. Dried and pulverized leaves are sold for making tea; also the leaf decoction is admistered as purgative for horses and used for the treatment of genetic urinary system.

Papaya fruits are widely consumed in food industry, while the processing of papaya fruits, papaya leaf is not consumed in any industry and lastly the papaya leaf dried and fallen from the papaya tree which will yield the garbage from the papaya tree. In order to solve such problem, study on the papaya leaf had been done. From the previous study done by Simmonne, (2005), papain enzyme from the papaya leaf yields the antibacterial and antifungal behaviour. As referring to research title, productions of antifungal hand wash and hand sanitizer by the natural enzyme are important for the bio green product which is more preferable than the chemical made product of hand wash and hand sanitizer. It is also environmental friendly and hazardless products which solve the garbage formation by papaya tree.

Papain enzyme can be obtained from papaya fruits, latex and roots. Papaya leaf has been chosen for the extraction for papain extraction because it provides the best potential for further commercial form. First, it is by-product of the papaya trees and low cost of raw materials which means we earn higher profit in selling our product. Papaya fruits consumed wisely in food industry, and it is wasted if the papaya fruit used only in extraction of papain enzyme since it contains a lot of nutrients and vitamins that beneficial to the consumer. While for papaya latex, the amount of latex produced by a papaya tree is not in large value, it is harder for us to extract the large amount of papain enzyme in the production of antifungal hand wash and hand sanitizer. Besides that, the cost for papain extraction from papaya latex is much higher compare with the extraction from papaya leaf. Papaya roots are the most important component for a papaya tree to survive. Once the roots are being cut, a papaya tree will die directly. It is a big waste of scarifying a papaya tree just for the papain enzyme extraction by papaya roots. As a conclusion, papaya leaf is the best choice and most preferable materials used for the papain enzyme extraction.

Normally, papain enzyme is obtained by extraction process. By mixing the chemical or physical or mechanical is the process of extraction. The extraction methods are Subcritical Water Extraction (SCWE), Microwave-Assisted Extraction (MAE), Soxhlet extraction(SE), Supercritical Fluid Extraction (SCFE), Pressurized Solvent Extraction (PSE) and Hot Water Extraction (HWE). All these method are applicable for papain enzyme extraction and for this research study, hot water extraction method is chosen for the papain extraction.

The most preferable extraction method for papain enzyme extraction is hot water extraction. Hot water extraction is and extraction method which is easy to be applied by industry without requiring expensive extraction equipment. In order to have higher amount of extraction, pre-treatment and ultrasonication process applied before proceed with extraction process. This is because purpose of pre-treatment process is to breakdown the cell wall of the papaya leaves and ultrasonication is to disrupt the intracellular of the cell. In University Malaysia Pahang, we do have the equipment required for the hot water extraction. There are Daihan ultrasonic cleaner and BS-21 shaking water bath.

1.2 Motivation, problem statement and brief review

Many microbes are present in the intestinal tracts of humans and animals. These are known as fecal microorganism. Simmonne, (2005) claims that a person's hand arms, or fingers may contaminated with faecal microorganisms after using toilet. Papain enzyme from the papaya leaf yields the antibacterial and antifungal behaviour, the papain which is a sulfhydryl protease is one of the most commonly used enzymes in various industries including food, tanning and pharmaceutical industries (Prakash et al., 2009). A study by Kamalkumar et al., (2007) shows that the papain has been used in meat tenderizers and in face and hair care products. It is also increasingly being used in pharmaceutical preparations and in such diverse manufacturing applications as leather, wool, rayon and beer. With the evidence supported, papain enzyme plays antifungal and antibacterial role, it could be functional as bio green soap, hand wash and hand sanitizer which enable to remove and inhibit the growth of the fungi and bacteria.

There are several choices of selection in making the, hand wash and hand sanitizer, for example, aloe vera, peach, lemon and some other plant. In Malaysia, papaya is the common plant, which could be seen in every state. By referring to Edison Frod (2011), to successfully grow a papaya tree, a frost free climate, lots of sunlight, lots of water and good soil needed. It is suit to Malaysia climate. Papaya is fast growing plant because it can grow up with fruits within 6 to 12 months. By using the by-product of papaya tree, the papaya leaf is beneficial in business and environmental friendly compare to using the other fruits such as, peach and lemon. In addition, peach could not plant in Malaysia, we have to import from the other country, such as China. For lemon, it had been used wisely in food industry; it could be harder in getting the large amount of lemon in producing the other product. On the other hand, only papaya fruits being consumed, papaya leaf, the by-product of papaya tree usually not being used in any industry and died all the time. It could be easy in harvesting the large amount of papaya leaf in extraction of papain enzyme for making the antifungal products. Based on the traditional use of the papaya leaf by Neuwinger, (2000), it is used as treatment for numerous maladies, ranging from gastrointestinal disorder to asthma and also as anthelmintic.

1.3 Objective

The following are the objectives of this research:

- Extract papain enzyme from papaya leaf
- Analyse the papain enzyme
- Study antifungal and antibacterial properties of papain enzyme
- Application of papain enzyme in production of antifungal and antibacterial handwash and hand
  sanitizer.

1.4 Scope of research

In order to achieve the objectives of this research study, several scope of study had been done. The scope in this research study is to make use of the waste disposal of papaya leaf which might cause the environmental problems. Throughout this research study, we have study the benefit of using the papaya leaf and also the papain enzyme's characteristic. In addition, we also study the extraction method of papain enzyme by hot water extraction. After that, we have to analyse the product of the extraction by Folin and Ciocalteu method to ensure that the product of extraction is papain enzyme.

Once we done the process of extraction, we will undergo antifungal test with *saccharomyces cerevisiae, Mucor spp and Rhizhopus spp.* species and antibacterial test with *staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* species. With this antifungal and antibacterial test, we can prove that papain enzyme is an antifungal and antibacterial enzyme. Next, we will apply the papain in making handwash and hand sanitizer. Apart from that, applications of antifungal papain enzyme in hand wash n hand sanitizer making could be done through this research study.

1.5 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a description of the applications of papain enzyme and general fact of papaya leaf, papain enzyme, hot water extraction and Folin and Ciocalteu method. A general description of antifungal test is presented. This chapter also provides the calibration curve of papain enzyme and a brief discussion of the advanced experimental techniques available for hot water extraction and Folin and Ciocalteu method their applications and limitations for papain analysis. A summary of the previous experimental work on papaya leaf extraction is also presented. A brief discussion on the methods for handwash and hand sanitizer making are also provided.

Chapter 3 gives a review of the extraction, enzyme assay, antifungal test, handwash and hand sanitizer procedures. The procedures start with the sample preparation then follow by the pre-treatment process, ultrasonication then lastly by hot water extraction for the extraction part. For the Enzymatic assay, we using the Folin and Ciocalteu method, then follow by using UV spectrophotometer. Then, the experimental data are collected and compare with the calibration curve. Once the enzymatic assay was done, we proceed with the antifungal test for papain enzyme to study the antifungal property of papain enzyme. Lastly, papain enzyme is used to make antifungal handwash and hand sanitizer. All the full description of each step is discussed in this chapter.

Chapter 4 is devoted to the result obtained. Discussion of the result obtained is presented in this chapter. A calculation of extraction yield is performed in this chapter. Experiment data of the enzymatic assay are preform and comparison of the experimental data with the calibration curve is done in this chapter. Antifungal test on the different of the diameter growth of the fungal is shown and discussion made.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This paper presents the experimental studies of papain enzyme property and the application of papain enzyme in making handwash and hand sanitizer. All the properties, specificity, characteristic, stability, structure, calibration curve, and setting of papain are discussed in literature review.

2.2 Introduction

Pawpaw (*Carica* papaya L.) is the most economically important fruit in the *Carica*ceae family (Oliver-Bever, 1986). Originally papaya is derived from the southern part of Mexico, *Carica* papaya is a perennial plant, and it is presently distributed over whole tropical area. It is an erect fast growing and usually unbranched tree or shrub. Although it is native to Central America, it has been transported to many parts of the tropics (Samson, 1986). The ripe fruit of the pawpaw plant is commonly consumed as food in different parts of the world. However, the unripen fruit is used as mild laxative, for diuresis, as galactogogue and as an abortifacient agent (Gill, 1992). Many parts of the plant are employed in the treatment of several ailments; for example the seed is used for expelling worms, and the seed and the roots are also used as abortifacient agent. The leaves (especially fallen ones) are used variously for the treatment of fever, pyrexia, diabetes, gonorrhoea, syphilis, inflammation and as dressing for foul wounds (Gill, 1992). Some of the scientifically validated uses of *Carica* papaya include the abortifacient activity of the seeds (Oderinde et al., 2002), the effects of the seeds on germinal epithelium of the seminiferous tubules (Uche-Nwachi et al., 2001), the fruit juice for lowering blood pressure (Eno et al., 2000), the wound healing effects of the leaves (Starley et al., 1999; Mikhal'chik et al., 2004), and several other studies.

Figure 2-1 Papaya tree

2.3 Papaya Leaf

Pawpaw (*Carica* papaya L.) is the most economically important fruit in the *Carica*ceae family (Oliver-Bever, 1986). It is an erect fast growing and usually unbranched tree or shrub. Although it is native to Central America, it has been transported to many parts of the tropics (Samson, 1986). Gill, (1992) claims that the leaves, especially fallen ones are used variously for the treatment of fever, pyrexia, diabetes, gonorrhoea, syphilis, inflammation and as dressing for foul wounds.

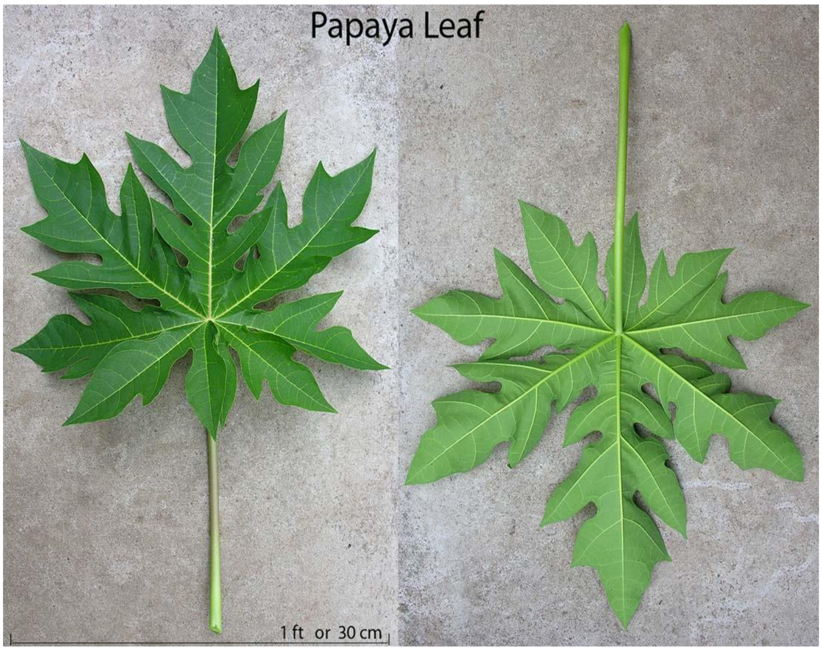

Figure 2-2 Papaya leaves

2.4 Cell membrane

Cell membrane is a biological membrane that separates the interior of the cells from the outside environment. It is selectively permeable to ions and organic molecules which control the movement of substances pass through the membrane. (Albert et al., 2002). The surrounding of the cell membrane is surrounded by phospholipid bilayer with embedded proteins. By referring to Belter, Cussler, & Wei-Shou., (1988), the basic envelop for Gram-negative cell, shown in the figure below, has three layers. The outer layer, about 8nm thick, consists of a polymer containing both protein and lipopolysaccharide. The second thinner layer, of peptidoglycan, exists in one form or another in virtually all species. Below this second layer is a gap, called the periplasmic space, which is also 8 nm thick. Enzymes are often located in this gap.

Gram-positive procaroytes are missing the first outer layer, but have both second peptidolycan layer and the periplasmic space. The third membrane, called the plasma membrane or the inner membrane is common to both Gram-positive and Gram-negative organisms. It consists largely of phospholipids, but also contains dispersed protein and metal ions. These lipid molecules have two parts, a hydrophobic part and hydrophilic part.

These three layers have different functions. The outer membrane and the peptidoglycan layer provide mechanical strength; it is their rupture. The weaker plasma membrane, the innermost layer controls the permeability of the cell, including transport of nutrients into the cell's interior and export of metabolites into the surrounding solution.

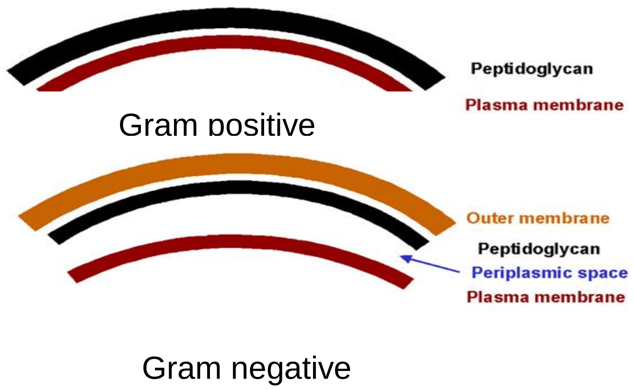

Figure 2-3 A schematic picture of cell wall by Fox, A., (2011)

2.5 Enzyme

Enzymes are protein in nature. Malvee, (2007) stated that, enzyme can be extracted from living tissues, purified and crystallized. Under controlled condition of isolation they retain their original level of activity and in some cases exhibit increased activity. Consequently purified enzyme can be used to carry out biochemical reaction outside the cell. This property of enzymes can be used in laboratory experiments and for commercial production of several important biochemical compound, drugs and industrial products. Therefore, enzyme research is an important area of biotechnology. For this research study the enzyme that we are going to study is papain enzyme which extracted from the papaya leaves.

2.6 Papain

Papain is a plant proteolytic enzyme for the cysteine proteinase family cysteine protease. Papain is found naturally in papaya (*Carica* papaya L.) manufactured from the latex of raw papaya fruits. Cohen et al. (1986) stated that it is very stable even at elevated temperature. Amri and Mamboya, (2012) stated that papain is able to break down organic molecules made of amino acids, known as polypeptides and thus plays a crucial role in diverse biological processes in physiological and pathological states, drug designs, industrial uses such as meat tenderizers and pharmaceutical preparations.

Papain was a highly active endolytic cysteine protease from *Carica* papaya. It is stable in harsh conditions and active at low and high temperature. It also is less expensive than microbial enzymes beside has wide range of specificity and good thermal stability amongst other proteases. With such unique characteristics, papain has potential used in detergents. Papain can be chemically modified by different dicarboxylic enhydrides of citraconic, phthalic, maleic and succinic acids as Lysine residues are not a part of active site in papain. Abraham & Sangeetha, (2006) claims these anhydrides react with the ε-amino group of lysine residues and change its charges from positive to negative, leading to a shift in pH optima of the enzyme from 7 to 9.

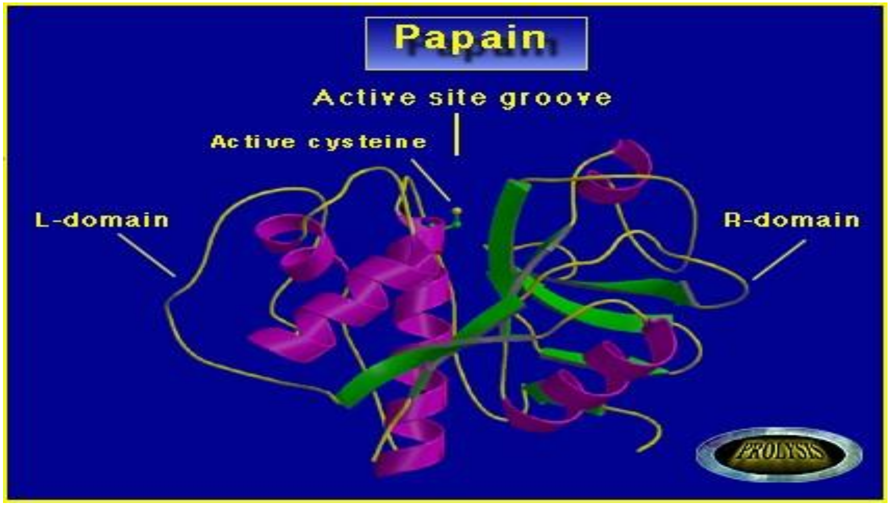

Figure 2-4 Papain structure by Calvero, (2007)

2.6.1 Papain specify

Papain has fairly broad specificity. It has endopeptidase, amidase and esterase acivities. Schechter and Berger, (1967) claims the active site consists of seven subsites (S1-S4 and S1’-S3’) that can each accommodate one amino acid residue of a substrate (P1-P4 and P1’- P3’). Specificity is controlled by S2 subsite, a hydrophobic pocket that accommodates the P2 side chain of the substrate. Papain exhibits specific substrate preferences primarily for bulky hydrophobic or aromatic residues at this subsite. (Kimmel and Smith, 1954). Outside the S2 subsite preferences, there is a lack of clearly defined residue selectivity.

2.6.2 Papain composition

Papain is a single-chained polypeptide with three disulphide bridges and a sulfhydryl group necessary for the activity of the enzyme. It is expressed as an inactive precursor, prepropapain. By referring to Vernet et al. (1995) the formation of active papain requires several cleavage steps including an initial cleavage of the 18 amino acid preregion (the signal sequence), follow by further cleavage of the glycosylated 114 amino acid proregion.

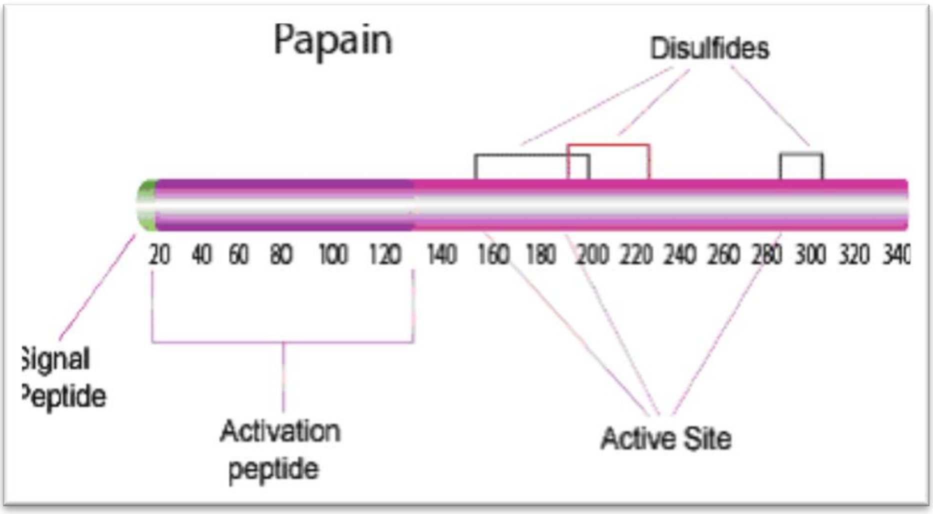

Figure 2-5 Papain composition by Margossian & Lowey, (1973)

2.6.3 Molecular Characteristics of papain

Azarkan et al., (2003) mention that mature forms of all papaya proteinases are between 212 and 218 amino acids, and exhibit a strong degree of homology. Maes et al., (1996) claims that X-ray structure analysis has shown that they adopt identical three dimensional folds.

2.6.4 Papain property

Protein Accession Number: P00784

CATH Classification:

- Class: Alpha Beta
- Architecture: Alpha Beta Complex
- Topology: Cathepsin B; Chain A

Molecular weight: 23.4kDa (Theoretical)

Optimal pH: 6.0- 7.0

Isoelectric Point: 8.88 (Theoretical)

Extinction Coefficient:

- 53610 cm-1M-1
- E1%, 280 = 22.88 (Theoretical)

Active Site Residues:

- Cysteine (C158)
- Histidine (H292)
- Asparagine (N308)

Activators:

- Cysteine
- Sulphide and sulphite
- Heavy metal chelating agents like EDTA
- N-bromosuccinimide

Inhibitors:

- PMSF
- TLCK, TPCK
- alpha2-macroglobulin
- Hg+ and other heavy metals
- AEBSF
- Antipain
- Cystatin
- E-64
- Leupeptin
- Sulfhydrl binding agents

•	Carbonyl reagents

•	Alkylating agent

All the papain properties adopt from Rozman-Pungercar et al., (2003)

Following are the data of papain activity toward pH, temperature and substrate concentration.

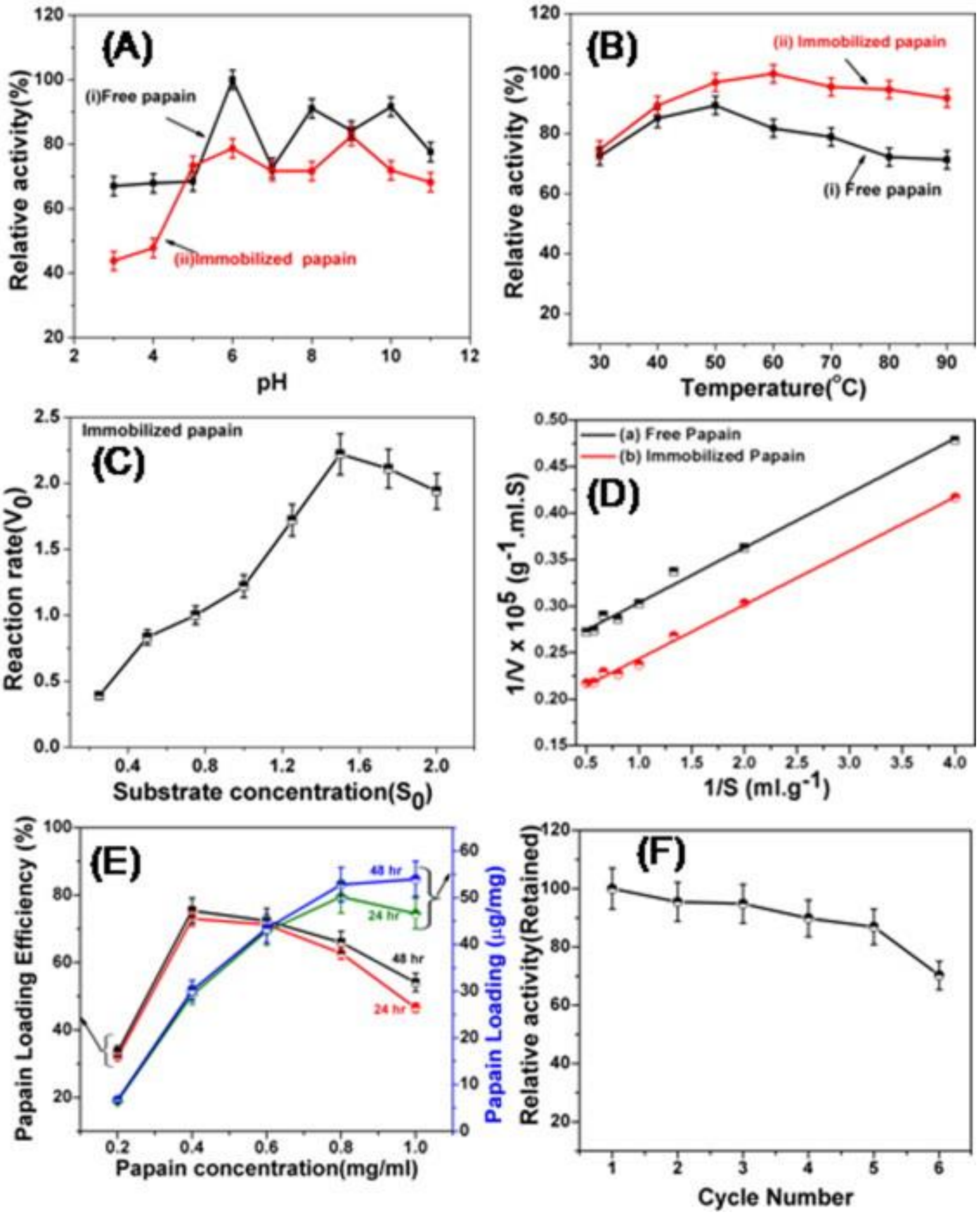

Figure 2-6 Papain activity toward parameters by Sahoo et al., (2013)